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Fat and *K-ras* mutations in sporadic colorectal cancer in The Netherlands Cohort Study

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Associations between dietary intake of various fats and specific *K-ras* mutations in colorectal cancer (CRC) were investigated within the framework of The Netherlands Cohort Study on diet and cancer (NLCS). After 7.3 years of follow-up and with exclusion of the first 2.3 years, 448 colon and 160 rectal cancer patients and 2948 subcohort members (55–69 years at baseline) were available for data-analyses. Mutation analysis of the *K-ras* gene was performed on all archival colon and rectal adenocarcinoma specimens. Case-cohort analyses were used to compute adjusted incidence rate ratios (RR) and 95% confidence intervals (CI) for colon and rectal cancer cases and for *K-ras* mutation subgroups. The intake of total, saturated and monounsaturated fat was not significantly associated with colon or rectal cancer. High intake of dietary polyunsaturated fat and, specifically, linoleic acid is associated with an increased risk of mutated *K-ras* colon tumours. The RRs for 1 SD of increase of polyunsaturated fat and linoleic acid were 1.21 (95% CI 1.05–1.41) and 1.22 (95% CI 1.05–1.42), respectively, and similar associations were observed for both G > A transitions and G > T or G > C transversions in the colon. In contrast, no significant associations were observed with rectal cancer risk, overall nor with specific *K-ras* mutation status. A high intake of polyunsaturated fat, in particular linoleic acid, may be an important dietary risk factor for *K-ras* mutated colon tumours, possibly by generating G > A transitions or G > T or G > C transversions in the *K-ras* oncogene.

Introduction

The current epidemiological evidence for the association of total fat and specific fatty acids with colorectal cancer (CRC) risk is controversial (1,2). The observed inconsistencies could, in part, be due to the heterogeneity of the colon and rectal cancer endpoint that is studied. Associations may become more apparent when the molecular events involved in colorectal carcinogenesis are taken into account.

The majority of colon and rectal tumours develop from small adenomatous polyps through a well-defined sequence of cytological and morphological changes (3), a process that is associated with the acquisition of somatic mutations (4,5). A genetic alteration that occurs in adenomas (10%) as well as carcinomas (40%) in colon and rectal cancer is the oncogenic activation of the *K-ras* gene by mutations. Activating mutations are mainly found in codons 12 and 13 (4,6–8). The most frequently observed types of point mutations are G > A transitions (8,9), and G > T and G > C transversions (10).

The link between fat intake and the pattern of mutations in human colon and rectal cancer is not clear. Only a few epidemiological studies have been conducted up to date on the association between the intake of fat and *K-ras* mutation status (11–14) and results are inconsistent. Experimental studies suggest that peroxidation of ω-6 polyunsaturated fatty acids (PUFAs) could lead to the accumulation of by-products like malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These compounds could react with DNA to form adducts, mainly the pyrimidopurinone adduct of deoxyguanosine (M_1G) (15–17). The presence of the M_1G adducts resulted predominantly in G > A and G > T, with very few G > C transversions in bacteria (16,18,19).

Consequently, exposures to specific dietary fat and fatty acids could contribute to the heterogeneity of acquired genetic alterations in the *K-ras* oncogene observed in colon and rectal tumours. Associations between dietary intakes of fat and specific fatty acids and the risk of specific point mutations in the *K-ras* oncogene in patients with colon and rectal cancer were studied within the framework of The Netherlands Cohort Study on diet and cancer (NLCS).

Materials and methods

Study population

The participants in this study are incident, colon and rectal cancer cases and subcohort members from the NLCS, which has been described in detail elsewhere (20). Briefly, the study was initiated in 1986 and includes 58 279 men and 62 573 women, aged 55–69 years old at baseline, who originated from 204 Dutch municipalities with computerized population registries. A self-administered questionnaire on diet and other risk factors for cancer was completed at baseline. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR, nine cancer registries in The Netherlands) and to PALGA, a nationwide network and registry of histo- and cytopathology (www.palga.nl) (21). In the municipalities included in the NLCS, the NCR and PALGA, together, have nearly 100% coverage since the start of the study (22–25). PALGA also provides necessary information on the identification of the pathology laboratory location of the storage of paraffin-embedded blocks of the eligible CRC patients. Accumulation of person-time in the cohort has been estimated through biennial vital status follow-up of a subcohort of 3500 men and women who were randomly selected after baseline exposure measurement (21). Cases with prevalent cancer other than non-melanoma skin cancer were excluded from the subcohort, which left 3346 men and women for analysis.

Abbreviations: CI, confidence intervals; CRC, colorectal cancer; MDA, malondialdehyde; M_1G, pyrimidopurinone–deoxyguanosine adducts; MUFA, monounsaturated fat; NLCS, The Netherlands Cohort Study; RR, rate ratios; PUFA, polyunsaturated fat.

The first 2.3 years of follow up were excluded due to incomplete coverage of PALGA alone in some of the municipalities included in the NLCS. Within this period, 83 subcohort members were either deceased or diagnosed with cancer other than non-melanoma skin cancer, leaving 3263 men and women for analysis. From 1989 until 1994, 929 incident cases with histologically confirmed CRC were observed of whom 819 could also be linked to a PALGA report of the lesion. The PALGA database was used to identify and locate tumour tissue in Dutch pathology laboratories. CRC was classified according to site as follows: colon, i.e. cecum through sigmoid colon (ICD-O-1 codes: 153.0, 153.1, 153.2, 153.3, 153.4, 153.5, 153.6, 153.7, 153.8, 153.9), rectosigmoid (ICD-O-1 code 154.0) and rectum (ICD-O-1 code 154.1). Information about age at baseline, sex and family history of CRC (at baseline) was retrieved from the NLCS database.

Tissue samples

This study is based on data of gene mutation analysis from CRC patients, described in detail elsewhere (8). Briefly, tumour material of all CRC patients was collected after approval by the Medical Ethics Committees of Maastricht University, the NCR and PALGA. Subsequently, all pathology laboratories in The Netherlands agreed to make relevant tissue samples available upon request from PALGA. Tissue samples of the 819 cases were distributed among 54 pathology laboratories throughout The Netherlands. Tumour tissue specimen collection started in August 1999 and was completed in December 2001. The loss to follow-up of tissue samples of cases amounted to 5%. Tissue samples from nine patients registered in one pathology laboratory could not be retrieved due to administrative inconsistencies, leaving 810 tissue samples for collection. For 34 cases, paraffin-embedded material was not available in the archives of pathology laboratories, leaving 776 cases for the determination of the *K-ras* mutation status. For 39 cases (5%), the *K-ras* mutation status could not be determined, i.e. for 20 cases only normal colonic mucosa was available, 10 cases were revised with a benign adenoma instead of an adenocarcinoma, for six cases the yield of DNA was not sufficient enough to determine *K-ras* mutation status and for three cases the available tissue did not include malignant CRC tissue. Finally, tumour material from 737 incident colorectal adenocarcinoma cases was available of whom 476 were colon cancer cases, 85 were rectosigmoid cancer cases and 176 were rectal cancer cases. Statistical analyses were performed separately for colon and rectal cancer as differences in the aetiology of colon and rectal cancer have been reported (1). Since the rectosigmoid can be considered as a clinically applied term rather than an anatomically defined transitional zone between the colon and rectum, patients with a rectosigmoid tumour were excluded from data analyses. Moreover, the number of patients with a rectosigmoid tumour was too small for adequate stratified analyses (8).

Detection of K-ras mutations

Mutation analysis of the exon 1 fragment of the *K-ras* oncogene, spanning codons 8–29, was performed on archival colorectal adenocarcinoma specimens of all 737 CRC patients using macrodissection, nested polymerase chain reaction (PCR) and direct sequencing of purified fragments, which has been described in detail elsewhere (8). The method of mutation detection was validated by the confirmation of reported *K-ras* status in CRC cell lines and a good correlation between fresh-frozen and routinely fixed, paraffin-embedded tissue. The detection limit was 5% mutated DNA. Duplo analyses revealed a good reproducibility (88%) (8). Evaluation of mutation analysis and data entry was independently performed by two observers (G.R. and M.L.).

The food frequency questionnaire

The dietary section of the questionnaire was a 150-item semi-quantitative food frequency questionnaire, which concentrated on habitual consumption of food and beverages during the year preceding the start of the study. Daily mean nutrient intakes were calculated using the computerized Dutch food composition table (26), by cumulating the multiplied frequencies and portion sizes of all food items with their tabulated nutrient contents. The questionnaire was validated against a 9-day diet record (27). Crude and energy-gender-adjusted (in parentheses) correlation coefficients were 0.72 (0.52) for total fat, 0.73 (0.58) for saturated fat and 0.73 (0.75) for PUFA (27). For energy intake the correlation coefficient was 0.74. On average, the questionnaire covered 91% of the record intake explaining part of the underestimation of energy intake from the questionnaire data (as presented in Table I). Questionnaire data were key-entered twice and processed for all incident cases in the cohort and for all subcohort members in a manner blinded with respect to case/subcohort status. This was done in order to minimize observer bias in coding and interpretation of the data.

For 257 subjects (28 incident colon adenocarcinoma cases, 16 incident rectal adenocarcinoma cases and 215 subcohort members, two subcohort members were also colon or rectal cancer cases), dietary data were incomplete or inconsistent, and they were excluded from the analyses. These subjects either (i) left 60 or more (out of 150) questionnaire items blank and ate fewer than 35 items at least once per month and/or (ii) left one or more item blocks (groups of items, e.g. beverages) blank. Additional details are given elsewhere (27). Hence, 608 colon and rectal cancer cases and 3048 subcohort members were available for data analyses.

Intake of specific fatty acids was based on a food composition database with specific fatty acids derived from the TRANSFAIR study (28). For this database, the hundred foods that contributed most to fat intake in the Dutch dietary pattern were sampled and analysed as methyl esters of the fatty acids present in the foods. In the database, total fat includes triglycerides and other lipids such as phospholipids and sterols. The percentage of triglycerides in total fat is assumed to be on average 93%, but varies across food sources. Daily intakes of total fat (g/day), saturated fat (g/day), monounsaturated fat (MUFA) (g/day), PUFA (g/day), and linolenic acid (g/day) and linoleic acid (g/day) as main constituents of PUFA were used as exposure variables. Linolenic and linoleic acid were used as the most abundant sources of ω-3 PUFAs and ω-6 PUFAs. For data-analyses, quartiles of the intake of fat and fatty acids were computed based on the distribution of subcohort members. Daily intake of dietary fibre (g/day), alcohol (g/day), fruit (g/day), vegetables (g/day) and total energy (kcal/day) and age at baseline (years), sex (men/women), Quetelet Index (QI; kg/m^2), physical activity (<30 min/day, 30–60 min/day, 60–90 min/day, >90 min/day), family history of CRC (yes/no) and smoking status (never/ex/current) were regarded as potential confounders.

Statistical analysis

The overall frequency of *K-ras* mutations as well as the type of mutation was computed for all colon and rectal cancer cases as described elsewhere (8). Fat intake was adjusted for energy by the residual method (29). Mean values of the continuous variables age at baseline (years), intake of total fat, saturated fat, MUFA, PUFA, linolenic acid, linoleic acid, dietary fibre, alcohol, fruit, vegetables and total energy and QI were evaluated for subcohort members and colon and rectal cancer cases with wild-type and mutated *K-ras* gene. Distributions in the categorical variables sex, family history of CRC, smoking status and physical activity were evaluated for subcohort members and colon and rectal cancer patients with wild-type and mutated *K-ras* gene. Differences in mean values of the continuous variables and the distributions in categorical variables between patients with wild-type and mutated *K-ras* tumours were tested with the Student's *t*-test and χ^2-test, respectively, using the statistical software package SPSS (version 9).

Incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) for colon and rectal cancer cases with wild-type or mutated *K-ras* gene tumours were estimated according to intakes of quartiles (with the lower quartile of intake regarded as the reference group) and of 1 standard deviation (SD) of increase of intake of total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid using Cox proportional hazards models with the STATA statistical software package (intercooled STATA, version 7). The total person-years at risk, estimated from the subcohort, were used in the analyses (27). Standard errors were estimated using the robust Hubert–White sandwich estimator to account for additional variance introduced by sampling from the cohort. This method is equivalent to the variance–covariance estimator as presented by Barlow (30). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (31). Using the backwards stepwise procedure, all confounders were tested in the overall colon and rectal cancer models separately. Interactions between fat and specific fatty acid intake and sex were tested for colon and rectal cancer separately and never found to be statistically significant. Therefore, results for men and women are presented together. Finally, age at baseline, sex, family history of CRC, smoking status, QI and the intake of energy were confounders for either one or both of the models and were therefore included as covariates for all models to be tested. Since 100 subcohort members had missing values for QI, results in the tables will be presented for 2948 subcohort members. For each analysis, trends were evaluated with the Wald test by fitting ordinal exposure variables (quartiles of intake) as continuous terms.

Results

The overall frequency and spectrum of mutations in the *K-ras* gene have been presented in detail elsewhere (8). In brief, a total of 227 mutations were found in 218 (36%) out of 608 colon and rectal cancer patients. The most frequently observed mutations are the G > A transitions (54%), G > T transversions (33%) and G > C transversions (7%). The observed frequencies of the point

Table I. Nutrient intake (mean ± SD) and other characteristics of the study population at baseline

	Subcohort	Colon cancer		*P*-value[a]	Rectal cancer		*P*-value[a]
		K-ras$^-$	K-ras$^+$		K-ras$^-$	K-ras$^+$	
n	2948	297	151		93	67	
Sex (% men)	48.2	51.5	58.9	0.14	72.0	55.2	0.03
Age (years)	61.3 ± 4.2	62.7 ± 4.0	63.8 ± 4.1	0.005	62.6 ± 4.1	62.2 ± 4.0	0.55
Fat variables*							
Total fat (g/day)	83.8 ± 15.8	84.3 ± 15.4	86.7 ± 14.8	0.12	86.7 ± 15.3	86.0 ± 14.2	0.76
Saturated fat (g/day)	33.2 ± 7.5	33.5 ± 6.9	33.6 ± 6.7	0.89	34.1 ± 6.8	34.3 ± 6.9	0.87
MUFA (g/day)**	31.4 ± 7.0	31.7 ± 6.5	32.1 ± 6.9	0.54	32.2 ± 6.6	32.5 ± 5.6	0.80
PUFA (g/day)***	17.3 ± 7.5	17.2 ± 7.3	19.3 ± 7.6	0.006	18.5 ± 8.9	17.5 ± 8.2	0.46
Linolenic acid (g/day)	1.3 ± 0.6	1.2 ± 0.5	1.3 ± 0.6	0.18	1.2 ± 0.6	1.3 ± 0.5	0.41
Linoleic acid (g/day)	16.0 ± 7.5	16.1 ± 7.3	18.0 ± 7.7	0.009	17.4 ± 8.9	16.2 ± 8.3	0.41
Other dietary factors							
Fibre (g/day)	27.0 ± 8.2	26.7 ± 7.6	27.7 ± 8.8	0.02	27.8 ± 8.0	27.6 ± 7.8	0.87
Alcohol (g/day)[b]	10.1 ± 14.1	11.0 ± 15.4	10.8 ± 14.2	0.59	13.8 ± 17.6	11.2 ± 12.2	0.30
Fruit (g/day)	177.0 ± 118.0	172.7 ± 123.7	176.9 ± 122.2	0.73	184.3 ± 145.8	180.7 ± 129.9	0.87
Vegetable (g/day)	193.8 ± 82.2	183.3 ± 78.2	198.0 ± 87.0	0.07	192.9 ± 72.4	188.0 ± 110.6	0.74
Energy (kcal/day)	1919.1 ± 517.1	1916.5 ± 494.4	1902.7 ± 472.9	0.78	2027.3 ± 517.4	1997.0 ± 449.1	0.70
Other characteristics							
QI (kg/m^2)	25.1 ± 3.1	25.5 ± 3.2	25.8 ± 3.3	0.42	24.9 ± 2.8	25.5 ± 2.9	0.20
Family history of CRC (% yes)	5.7	13.5	9.3	0.20	9.7	11.9	0.66
Smoker (%)							
Never	36.9	36.7	37.1		25.8	34.3	
Ex-smoker	35.2	43.1	46.4		41.9	43.3	
Current smoker	27.8	20.2	16.6	0.62	32.3	22.4	0.31
Physical activity (%)[c]							
<30 min/day	20.1	19.7	20.3		18.5	22.7	
30–60 min/day	32.2	33.7	32.4		27.2	27.3	
60–90 min/day	31.1	29.6	29.1		32.6	34.8	
>90 min/day	15.4	17.0	18.2	0.98	21.7	15.2	0.73

[a]Comparing cases with at least one *K-ras* mutation to cases without a *K-ras* mutation.
[b]For alcohol intake the mean levels in the subcohort are based on 2862 subjects.
[c]For physical activity the mean levels in the subcohort are based on 2915 subjects.
*Adjusted for energy by the residual method (29).
**Monounsaturated fat.
***Polyunsaturated fat.

mutation are similar to the frequencies of the 737 colorectal cancer cases, including rectosigmoid cancer cases, for whom *K-ras* status was determined (8).

Table I shows various types of fat intake and other baseline characteristics of the study population. Colon and rectal cancer cases were more often men, were older, more frequently reported a family history of colorectal cancer and had a higher daily alcohol intake as compared with the subcohort. Colon cancer cases with a mutated *K-ras* tumour were significantly older, had higher daily intakes of PUFA, linoleic acid, fibres and vegetables than colon cancer cases with a wild-type *K-ras* tumour. There were no statistically significant differences between colon cancer cases with a wild-type *K-ras* tumour and a mutated *K-ras* tumour in dietary intakes of total fat, saturated fat, MUFA, linolenic acid and other factors presented in Table I. Rectal cancer cases with a mutated *K-ras* tumour were less frequently men as compared with rectal cancer cases with a wild-type *K-ras* tumour. No statistically significant differences between rectal cancer cases with mutated *K-ras* tumours and wild-type *K-ras* tumours were observed for total fat, specific fatty acids and other (dietary) factors.

Associations between the intake of total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid with the risk of colon or rectal cancer are presented in Table II. Incidence RR and 95% CI for colon and rectal cancer are adjusted for age and sex and for age, sex, smoking, QI, energy intake and family history of CRC. The age–sex adjusted RR and the multivariate RR were similar. Frequent consumption of total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid were all not associated with the risk of colon or rectal cancer (Table II).

Associations of total fat, saturated fat, MUFA, PUFA, linolenic and linoleic acid with wild-type *K-ras* tumours and mutated *K-ras* tumours in the colon or rectum are presented in Table III. Results will first be discussed for wild-type *K-ras* tumours. No clear associations were observed for high intakes of total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid and the risk of colon and rectal cancer with a wild-type *K-ras* gene. With regard to the colon mutated *K-ras* tumours, positive associations were observed for high intakes of PUFA (RR for highest versus lowest quartile of intake 2.09, 95% CI 1.25–3.49, P_{trend} 0.002) and specifically linoleic acid (RR for highest versus lowest quartile of intake 2.19, 95%CI 1.30–3.67, P_{trend} 0.002), but not linolenic acid. These positive associations were also observed for 1 SD of increase of intake (Table III). No significant associations were observed for rectal mutated *K-ras* tumours and for highest versus lowest intakes of total fat, saturated fat, MUFA and linolenic acid and colon and rectal cancer with mutated *K-ras* tumours.

Subgroup analyses were performed to evaluate the associations between total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid intakes and specific types of *K-ras* point mutations (G > A transitions and G > T or G > C transversions)

Table II. Incidence RR and 95% CI for colon and rectal cancer patients (448 colon and 160 rectal cancer cases) according to the intake of total fat and specific fatty acids

Exposure*	Quartile of intake					RR (95% CI) for 1 SD increase in intake#
	Q1[a]	Q2	Q3	Q4	*P* for trend	
Total fat						
Median intake (g/day)						
Men	78.1	90.3	98.5	109.1		
Women	63.0	71.5	77.6	85.3		
Colon cases/rectal cases/person-years	113/43/3657	126/42/3675	95/34/3741	114/40/3664		
RR (95% CI)[b]						
Colon	1.00	1.09 (0.82–1.43)	0.80 (0.60–1.08)	0.99 (0.74–1.32)	*0.49*	1.02 (0.90–1.15)
Rectum	1.00	0.96 (0.62–1.49)	0.75 (0.47–1.20)	0.92 (0.59–1.44)	*0.52*	0.94 (0.78–1.13)
RR (95% CI)[c]						
Colon	1.00	1.09 (0.82–1.44)	0.74 (0.55–1.01)	0.95 (0.71–1.27)	*0.28*	1.00 (0.88–1.13)
Rectum	1.00	0.89 (0.56–1.39)	0.74 (0.46–1.19)	0.86 (0.55–1.36)	*0.41*	0.91 (0.76–1.09)
Saturated fat						
Median intake (g/day)						
Men	28.9	33.7	38.5	45.8		
Women	23.9	27.8	31.0	36.6		
Colon cases/rectal cases/person-years	101/43/3645	123/35/3703	119/46/3709	105/36/3679		
RR (95% CI)[b]						
Colon	1.00	1.20 (0.90–1.59)	1.14 (0.85–1.51)	0.97 (0.72–1.30)	*0.72*	0.98 (0.89–1.09)
Rectum	1.00	0.80 (0.50–1.26)	1.04 (0.67–1.59)	0.80 (0.51–1.26)	*0.58*	0.97 (0.83–1.15)
RR (95% CI)[c]						
Colon	1.00	1.19 (0.88–1.60)	1.10 (0.82–1.49)	0.93 (0.69–1.26)	*0.51*	0.97 (0.87–1.08)
Rectum	1.00	0.79 (0.49–1.28)	1.02 (0.65–1.59)	0.73 (0.46–1.17)	*0.37*	0.95 (0.80–1.12)
MUFA**						
Median intake (g/day)						
Men	28.2	33.2	36.9	42.5		
Women	22.4	26.1	28.9	33.0		
Colon cases/rectal cases/person-years	101/45/3660	124/34/3650	119/41/3714	104/40/3713		
RR (95% CI)[b]						
Colon	1.00	1.23 (0.92–1.63)	1.17 (0.88–1.56)	1.03 (0.76–1.37)	*0.97*	1.00 (0.89–1.11)
Rectum	1.00	0.76 (0.48–1.21)	0.91 (0.59–1.41)	0.89 (0.57–1.38)	*0.77*	0.93 (0.79–1.10)
RR (95% CI)[c]						
Colon	1.00	1.19 (0.89–1.60)	1.12 (0.82–1.51)	0.98 (0.73–1.33)	*0.77*	0.99 (0.88–1.12)
Rectum	1.00	0.70 (0.44–1.13)	0.91 (0.57–1.44)	0.87 (0.56–1.37)	*0.80*	0.93 (0.78–1.11)
PUFA***						
Median intake (g/day)						
Men	11.6	16.0	21.0	29.1		
Women	8.8	12.4	16.2	22.5		
Colon cases/rectal cases/person-years	95/47/3656	124/33/3683	113/39/3726	116/41/3667		
RR (95% CI)[b]						
Colon	1.00	1.35 (1.01–1.81)	1.23 (0.92–1.66)	1.24 (0.92–1.66)	*0.26*	1.05 (0.96–1.16)
Rectum	1.00	0.71 (0.45–1.13)	0.84 (0.54–1.30)	0.87 (0.56–1.34)	*0.68*	1.00 (0.84–1.19)
RR (95% CI)[c]						
Colon	1.00	1.38 (1.02–1.86)	1.24 (0.91–1.68)	1.21 (0.89–1.64)	*0.37*	1.03 (0.94–1.14)
Rectum	1.00	0.74 (0.46–1.19)	0.86 (0.55–1.34)	0.83 (0.53–1.29)	*0.54*	0.98 (0.83–1.16)
Linolenic acid						
Median intake (g/day)						
Men	0.8	1.2	1.5	2.0		
Women	0.6	0.9	1.2	1.6		
Colon cases/rectal cases/person-years	108/44/3642	108/40/3684	124/36/3657	108/40/3750		
RR (95% CI)[b]						
Colon	1.00	0.97 (0.73–1.30)	1.15 (0.87–1.52)	1.01 (0.76–1.35)	*0.65*	0.99 (0.90–1.09)
Rectum	1.00	0.89 (0.57–1.38)	0.81 (0.51–1.28)	0.90 (0.58–1.41)	*0.59*	0.94 (0.79–1.11)
RR (95% CI)[c]						
Colon	1.00	0.95 (0.70–1.30)	1.10 (0.82–1.47)	1.01 (0.75–1.36)	*0.70*	0.98 (0.89–1.09)
Rectum	1.00	0.91 (0.57–1.47)	0.87 (0.55–1.38)	0.91 (0.58–1.44)	*0.67*	0.95 (0.80–1.12)
Linoleic acid						
Median intake (g/day)						
Men	10.0	14.8	19.5	27.9		
Women	7.4	11.2	15.0	21.2		
Colon cases/rectal cases/person-years	91/41/3658	126/40/3700	113/35/3699	118/44/3676		

Table II. continued

Exposure*	Quartile of intake					RR (95% CI) for 1 SD increase in intake#
	Q1[a]	Q2	Q3	Q4	*P* for trend	
RR (95% CI)[b]						
Colon	1.00	1.44 (1.07–1.92)	1.29 (0.96–1.73)	1.30 (0.97–1.75)	*0.17*	1.07 (0.97–1.18)
Rectum	1.00	0.98 (0.63–1.54)	0.86 (0.54–1.37)	1.06 (0.68–1.64)	*0.96*	1.02 (0.86–1.21)
RR (95% CI)[c]						
Colon	1.00	1.49 (1.10–2.02)	1.32 (0.97–1.80)	1.31 (0.97–1.77)	*0.19*	1.06 (0.96–1.17)
Rectum	1.00	1.03 (0.65–1.64)	0.90 (0.56–1.45)	1.03 (0.66–1.62)	*0.96*	1.00 (0.84–1.18)

[a]Reference category of quartiles of intake.
[b]Relative risk adjusted for age and sex.
[c]Relative risk adjusted for age, sex, QI, smoking, energy intake and family history of CRC.
*Adjusted for energy by the residual method (29).
**Monounsaturated fat.
***Polyunsaturated fat.
#See for 1 SD of increase Table I.

in colon and rectal tumours (Table IV). High intake of PUFA and specifically linoleic acid showed positive associations with colon tumours harbouring G > A transitions (RR for highest versus lowest quartile of intake 1.95, 95% CI 1.01–3.77 and RR 1.80, 95% CI 0.92–3.51, respectively) or G > T or G > C transversions (RR 2.17, 95% CI 0.98–4.80 and RR 2.69, 95% CI 1.19–6.08, respectively). These positive associations were also observed for 1 SD of increase of intake (Table IV). There were no associations found between PUFA and linoleic acid intake and rectal tumours with G > A transitions or G > T or G > C transversions. In addition, no significant associations were observed between highest versus lowest quartile of intake of total fat, saturated fat, MUFA and linolenic acid and the risk of G > A transitions or G > T or G > C transversions in colon and rectal tumours.

Discussion

In this study, no associations were observed between intakes of specific fats or fatty acids and overall colon and rectal cancer risk. However, associations were observed when taking into account the presence or absence of specific point mutations in the *K-ras* gene of colon and rectal cancer patients. No significant associations were observed for total fat, saturated fat, and MUFA intakes and the risk of colon and rectal tumours without or with a *K-ras* mutation. In contrast, positive associations were observed for high intake of PUFA, specifically linoleic acid but not linolenic acid, and the risk of colon tumours harbouring *K-ras* oncogene mutations. Subgroup analyses of specific point mutations in the *K-ras* gene revealed that these positive associations were observed for G > A transitions and G > T and G > C transversions.

This cohort study on fat and specific fatty acids intake in relation to specific *K-ras* mutations in colon and rectal cancer is, to our knowledge, the only prospective study performed to date. One large case-control study with colon cancer patients (11), two case-control studies of limited size among colon (13), and colon and rectal cancer patients (12) and one cross-sectional case–case study with colorectal adenoma patients (14) have been conducted previously. The prospective design of the current study and high completeness of follow-up of cancer incidence and subcohort, make information and selection bias unlikely. In addition, as a result of the exclusion of the first 2.3 years of follow-up, the chance of information bias due to potential pre-clinical colorectal cancer is minimal. Finally, tumour tissue was available for 84% of the eligible cases (776 out of 929) and there were no significant differences in fat intake levels nor in clinicopathological characteristics of the tumour (Dukes' stage and differentiation grade) of these patients compared with the CRC patients with a known *K-ras* mutation status of their tumour (data not shown). Therefore, selection bias due to loss to follow-up is unlikely.

In general, the validation of the fat intake variables was satisfactory (see Materials and methods section and ref. 27). Although total fat intake and energy intake may have been underestimated as compared with the 9-day record method in the validation study (27) and, in addition, there is inevitably a certain degree of misclassification of fat intake variables, it is not expected that this is differential with respect to the *K-ras* mutation status of the tumour. Therefore, the associations observed in this study are probably attenuated. In other epidemiological studies similar inaccuracies occur in the measurement of fat intake since similar methods of intake assessment were employed (11,12,14).

One of the hypotheses for the association between fat intake and colorectal cancer is that meat consumption instead of fat intake determines the increased risk. We also investigated meat consumption as a potential risk factor for *K-ras* mutations in colon and rectal cancer, and total fresh meat was not associated with risk. Therefore, we did not additionally adjust for this potential confounder in these analyses.

In the current study, a high intake of total and saturated fats was not associated with overall colon or rectal cancer risk and with *K-ras* mutation status and these findings are supported by others (11,13,14). Bautista *et al*. (12) observed an inverse association between a high intake of total fat and colorectal tumours with a *K-ras* wild-type gene (odds ratio for highest versus lowest tertile of intake 0.47, 95% CI 0.22–0.99). However, the results were based on only 108 colorectal cancer cases in a population-based case-control setting, and therefore estimates of associations must be interpreted with caution.

Animal studies indicated an inverse association of high MUFA intake, derived mainly from oleic acid with colorectal cancer risk (32,33). However, in epidemiological studies, no clear associations were observed with colorectal cancer risk (34–36). Regarding the *K-ras* mutation status, no associations

Table III. Adjusted RR[a] and 95% CI for colon and rectal cancer patients with wild type *K-ras* gene and with mutated *K-ras* oncogene according to the intake of total fat and specific fatty acids

Exposure*	Quartile of intake					RR (95% CI) for 1 SD increase in intake[#]
	Q1[b]	Q2	Q3	Q4	*P* for trend	
Total fat						
Cases						
Colon *K-ras*$^-$/*K-ras*$^+$	75/35	81/44	57/32	77/33		
K-ras$^-$/*K-ras*$^+$ rectum	25/18	26/13	21/12	16/22		
$RR_{K\text{-}ras^-}$ (95% CI)						
Colon	1.00	1.04 (0.74–1.45)	0.71 (0.49–1.02)	0.99 (0.70–1.38)	*0.48*	0.98 (0.84–1.13)
Rectum	1.00	1.00 (0.57–1.76)	0.80 (0.44–1.47)	0.63 (0.33–1.20)	*0.11*	0.81 (0.64–1.02)
$RR_{K\text{-}ras^+}$ (95% CI)						
Colon	1.00	1.19 (0.75–1.90)	0.82 (0.49–1.36)	0.88 (0.54–1.44)	*0.32*	1.03 (0.84–1.26)
Rectum	1.00	0.72 (0.35–1.51)	0.65 (0.31–1.37)	1.18 (0.63–2.23)	*0.65*	1.07 (0.81–1.41)
Saturated fat						
Cases						
Colon *K-ras*$^-$/*K-ras*$^+$	60/39	86/34	76/39	68/32		
K-ras$^-$/*K-ras*$^+$ rectum	25/18	21/13	26/18	17/16		
$RR_{K\text{-}ras^-}$ (95% CI)						
Colon	1.00	1.42 (0.99–2.02)	1.22 (0.84–1.75)	1.06 (0.74–1.54)	*0.96*	1.01 (0.89–1.15)
Rectum	1.00	0.82 (0.45–1.52)	1.02 (0.57–1.80)	0.64 (0.34–1.21)	*0.28*	0.86 (0.70–1.06)
$RR_{K\text{-}ras^+}$ (95% CI)						
Colon	1.00	0.85 (0.52–1.38)	0.92 (0.58–1.48)	0.73 (0.45–1.18)	*0.26*	0.90 (0.75–1.07)
Rectum	1.00	0.75 (0.35–1.59)	1.02 (0.52–2.01)	0.86 (0.43–1.72)	*0.89*	1.08 (0.85–1.39)
MUFA**						
Cases						
Colon *K-ras*$^-$/*K-ras*$^+$	61/37	81/41	73/40	75/26		
K-ras$^-$/*K-ras*$^+$ rectum	29/15	19/12	21/19	20/19		
$RR_{K\text{-}ras^-}$ (95% CI)						
Colon	1.00	1.27 (0.89–1.82)	1.16 (0.80–1.68)	1.18 (0.82–1.68)	*0.53*	1.01 (0.88–1.17)
Rectum	1.00	0.64 (0.35–1.17)	0.71 (0.39–1.28)	0.69 (0.38–1.24)	*0.27*	0.82 (0.64–1.06)
$RR_{K\text{-}ras^+}$ (95% CI)						
Colon	1.00	1.06 (0.66–1.70)	1.04 (0.64–1.69)	0.67 (0.40–1.13)	*0.14*	0.94 (0.77–1.16)
Rectum	1.00	0.81 (0.38–1.76)	1.31 (0.64–2.68)	1.22 (0.61–2.44)	*0.34*	1.10 (0.89–1.37)
PUFA***						
Cases						
Colon *K-ras*$^-$/*K-ras*$^+$	68/23	87/31	72/41	63/49		
K-ras$^-$/*K-ras*$^+$ rectum	24/21	18/14	25/14	22/16		
$RR_{K\text{-}ras^-}$ (95% CI)						
Colon	1.00	1.36 (1.00–1.91)	1.05 (0.73–1.49)	0.91 (0.63–1.31)	*0.32*	0.94 (0.83–1.07)
Rectum	1.00	0.76 (0.41–1.43)	1.03 (0.58–1.82)	0.89 (0.49–1.62)	*0.96*	0.97 (0.78–1.21)
$RR_{K\text{-}ras^+}$ (95% CI)						
Colon	1.00	1.44 (0.82–2.52)	1.83 (1.08–3.11)	2.09 (1.25–3.49)	*0.002*	1.21 (1.05–1.41)
Rectum	1.00	0.72 (0.36–1.45)	0.67 (0.33–1.34)	0.75 (0.39–1.44)	*0.38*	0.99 (0.77–1.27)
Linolenic acid						
Cases						
Colon *K-ras*$^-$/*K-ras*$^+$	69/38	71/33	79/38	71/35		
K-ras$^-$/*K-ras*$^+$ rectum	30/12	21/17	18/18	20/18		
$RR_{K\text{-}ras^-}$ (95% CI)						
Colon	1.00	1.02 (0.70–1.46)	1.16 (0.82–1.64)	1.05 (0.74–1.49)	*0.63*	0.95 (0.85–1.08)
Rectum	1.00	0.65 (0.36–1.18)	0.57 (0.32–1.04)	0.66 (0.37–1.17)	*0.14*	0.86 (0.70–1.11)
$RR_{K\text{-}ras^+}$ (95% CI)						
Colon	1.00	0.84 (0.50–1.41)	1.00 (0.62–1.60)	0.95 (0.59–1.55)	*0.97*	1.04 (0.88–1.23)
Rectum	1.00	1.57 (0.71–3.50)	1.62 (0.76–3.45)	1.54 (0.73–3.25)	*0.27*	1.07 (0.88–1.29)
Linoleic acid						
Cases						
Colon *K-ras*$^-$/*K-ras*$^+$	64/22	89/33	72/40	65/49		
K-ras$^-$/*K-ras*$^+$ rectum	20/19	26/13	20/15	23/18		
$RR_{K\text{-}ras^-}$ (95% CI)						
Colon	1.00	1.46 (1.03–2.06)	1.14 (0.79–1.63)	1.01 (0.70–1.45)	*0.63*	0.97 (0.86–1.10)
Rectum	1.00	1.32 (0.73–2.39)	0.99 (0.53–1.86)	1.12 (0.61–2.07)	*0.97*	0.99 (0.80–1.23)
$RR_{K\text{-}ras^+}$ (95% CI)						
Colon	1.00	1.60 (0.92–2.80)	1.87 (1.09–3.22)	2.19 (1.30–3.67)	*0.002*	1.22 (1.05–1.42)
Rectum	1.00	0.72 (0.34–1.51)	0.81 (0.40–1.63)	0.93 (0.48–1.79)	*0.91*	1.00 (0.77–1.29)

[a]Multivariate adjusted RR for age, sex, QI, smoking, energy intake and family history of CRC and their 95% CI.
[b]Reference category of quartiles of intake.
*Adjusted for energy by the residual method (29).
**Monounsaturated fat.
***Polyunsaturated fat.
[#]See for 1 SD of increase Table I.

Table IV. Adjusted RR[a] and 95% CI for colon and rectal cancer patients with G>A transitions or with G>T or G>C transversions in the *K-ras* oncogene according to the intake of total fat and specific fatty acids

Exposure*	Quartile of intake					RR (95% CI) for 1 SD increase in intake[#]
	Q1[b]	Q2	Q3	Q4	*P* for trend	
Total fat						
Cases						
$G>A^+$/ $G>T^+$, $G>C^+$						
Colon	20/15	23/20	17/12	22/9		
Rectum	12/7	6/7	6/7	9/11		
$RR_{G>A^+}$ (95% CI)						
Colon	1.00	1.11 (0.60–2.07)	0.78 (0.40–1.52)	1.02 (0.54–1.90)	*0.79*	1.09 (0.82–1.44)
Rectum	1.00	0.45 (0.17–1.22)	0.43 (0.16–1.19)	0.74 (0.31–1.79)	*0.50*	0.87 (0.57–1.32)
$RR_{G>T^+,G>C^+}$ (95% CI)						
Colon	1.00	1.26 (0.64–2.48)	0.72 (0.33–1.56)	0.57 (0.25–1.30)	*0.07*	0.90 (0.69–1.17)
Rectum	1.00	1.09 (0.37–3.20)	1.07 (0.37–3.11)	1.50 (0.58–3.89)	*0.42*	1.17 (0.84–1.63)
Saturated fat						
Cases						
$G>A^+$/ $G>T^+$, $G>C^+$						
Colon	23/14	16/17	23/15	20/10		
Rectum	9/10	5/8	13/3	6/11		
$RR_{G>A^+}$ (95% CI)						
Colon	1.00	0.69 (0.36–1.34)	0.95 (0.52–1.74)	0.76 (0.41–1.42)	*0.60*	0.95 (0.75–1.19)
Rectum	1.00	0.51 (0.17–1.55)	1.31 (0.55–3.10)	0.64 (0.23–1.84)	*0.88*	1.01 (0.70–1.45)
$RR_{G>T^+,G>C^+}$ (95% CI)						
Colon	1.00	1.19 (0.57–2.47)	1.00 (0.47–2.10)	0.63 (0.28–1.43)	*0.21*	0.79 (0.62–1.01)
Rectum	1.00	0.91 (0.34–2.44)	0.33 (0.08–1.25)	1.07 (0.45–2.55)	*0.84*	1.15 (0.80–1.64)
MUFA**						
Cases						
$G>A^+$/$G>T^+$, $G>C^+$						
Colon	19/17	21/18	25/13	17/8		
Rectum	10/4	6/7	7/13	10/8		
$RR_{G>A^+}$ (95% CI)						
Colon	1.00	1.09 (0.57–2.09)	1.34 (0.70–2.56)	0.84 (0.43–1.66)	*0.78*	1.05 (0.80–1.39)
Rectum	1.00	0.53 (0.19–1.46)	0.59 (0.22–1.66)	0.96 (0.40–2.33)	*0.99*	1.06 (0.71–1.60)
$RR_{G>T^+,G>C^+}$ (95% CI)						
Colon	1.00	1.01 (0.51–1.98)	0.72 (0.34–1.51)	0.46 (0.20–1.07)	*0.04*	0.78 (0.60–1.02)
Rectum	1.00	2.00 (0.57–7.07)	4.00 (1.25–12.81)	1.93 (0.57–6.56)	*0.12*	1.12 (0.91–1.37)
PUFA***						
Cases						
$G>A^+$/$G>T^+$, $G>C^+$						
Colon	14/9	18/13	23/14	27/20		
Rectum	11/10	6/9	11/4	5/9		
$RR_{G>A^+}$ (95% CI)						
Colon	1.00	1.44 (0.71–2.94)	1.76 (0.89–3.49)	1.95 (1.01–3.77)	*0.04*	1.18 (0.97–1.42)
Rectum	1.00	0.51 (0.18–1.43)	0.86 (0.35–2.11)	0.42 (0.14–1.20)	*0.21*	0.80 (0.57–1.12)
$RR_{G>T^+,G>C^+}$ (95% CI)						
Colon	1.00	1.51 (0.64–3.60)	1.57 (0.67–3.70)	2.17 (0.98–4.80)	*0.06*	1.24 (0.98–1.57)
Rectum	1.00	1.10 (0.44–2.74)	0.45 (0.14–1.46)	0.94 (0.38–2.30)	*0.59*	1.02 (0.72–1.45)
Linolenic acid						
Cases						
$G>A^+$/$G>T^+$, $G>C^+$						
Colon	24/14	13/18	24/14	21/10		
Rectum	6/6	8/9	9/11	11/6		
$RR_{G>A^+}$ (95% CI)						
Colon	1.00	0.54 (0.26–1.11)	1.02 (0.57–1.85)	0.91 (0.49–1.70)	*0.79*	1.04 (0.83–1.29)
Rectum	1.00	1.04 (0.33–3.28)	1.39 (0.47–4.04)	1.75 (0.62–4.95)	*0.22*	1.21 (0.92–1.59)
$RR_{G>T^+,G>C^+}$ (95% CI)						
Colon	1.00	1.26 (0.57–2.80)	1.00 (0.47–2.14)	0.75 (0.32–1.72)	*0.38*	0.95 (0.73–1.25)
Rectum	1.00	1.91 (0.64–5.66)	2.21 (0.81–6.03)	1.09 (0.35–3.38)	*0.75*	0.92 (0.71–1.20)

Table IV. continued

Exposure*	Quartile of intake					RR (95% CI) for 1 SD increase in intake#
	Q1[b]	Q2	Q3	Q4	*P* for trend	
Linoleic acid						
Cases						
$G>A^+/G>T^+$, $G>C^+$						
Colon	14/8	19/14	24/12	25/22		
Rectum	11/10	6/7	10/6	7/9		
$RR_{G>A^+}$ (95% CI)						
Colon	1.00	1.51 (0.75–3.07)	1.85 (0.93–3.66)	1.80 (0.92–3.51)	*0.06*	1.19 (0.98–1.44)
Rectum	1.00	0.56 (0.19–1.60)	0.89 (0.35–2.26)	0.65 (0.24–1.74)	0.59	0.81 (0.57–1.15)
$RR_{G>T^+,G>C^+}$ (95% CI)						
Colon	1.00	1.83 (0.76–4.40)	1.52 (0.61–3.77)	2.69 (1.19–6.08)	*0.03*	1.27 (1.00–1.61)
Rectum	1.00	0.83 (0.31–2.27)	0.70 (0.25–1.94)	0.94 (0.38–2.31)	*0.83*	1.06 (0.74–1.53)

[a]Multivariate adjusted RR for age, sex, QI, smoking, energy intake and family history of CRC and their 95% CI.
[b]Reference category of quartiles of intake.
*Adjusted for energy by the residual method (29).
**Monounsaturated fat.
***Polyunsaturated fat.
#See for 1 SD of increase Table I.

were observed for high intake of MUFA and the risk of colon and rectal cancer in the current study. This is in line with Slattery's observation (11). However, Bautista *et al*. (12) observed that a high intake of MUFA was inversely associated with the risk of having a wild-type *K-ras* gene. This study was conducted in Spain where the main source of oleic acid is olive oil. This is not the case for the current cohort and could be an explanation for the different findings. The association was not reported in the other studies (13,14).

To our best knowledge, this is the first study reporting on specific PUFAs in relation to *K-ras* mutation status. Overall PUFA intake was studied by Slattery *et al*. (11) and Bautista *et al*. (12) and no associations were observed with *K-ras* mutation status.

In the current study, no clear associations were observed between the high intake of linolenic acid as the main source of ω-3 PUFAs, and the risk of colon or rectal cancer with or without specific point mutations in the *K-ras* gene. Epidemiological, clinical and experimental data indicate a protective effect of fish oil-derived ω-3 PUFAs on overall colon cancer (35,37–44). Collett *et al*. (37) demonstrated that the major ω-3 PUFA constituent of fish oil, docosahexaenoic acid reduces the Ras protein localization to the plasma membrane without affecting post-translational lipidation and lowers the GTP-binding of the Ras protein in mouse colonocytes treated with azoxymethane (colon carcinogen). These findings were corroborated by *in vivo* studies (45). Another hypothesis is that the tumour-promoting activity of ω-6 PUFA was abrogated by competitive inhibition of ω-3 PUFA from the metabolism of arachidonic acid and therefore reducing MDA levels and subsequently $M_1G>T$ and $M_1G>C$ transversions (38). Summarized, ω-3 PUFAs may exert their effect through inhibition of the Ras protein activity and not by generating functional aberrations in the exon 1 fragment of the *K-ras* oncogene. This could explain the lack of association between linolenic acid and *K-ras* mutation status of colon or rectal tumours in this study.

In the current study, no significant association was observed for linoleic acid, as the main source of ω-6 PUFAs intake, and the overall risk of colon or rectal cancer. This is in line with most epidemiological evidence up to date (46). However, a positive effect was found between high intake of linoleic acid and colon tumours harbouring *K-ras* gene mutations. Subgroup analyses of specific point mutations in the *K-ras* gene revealed that colon tumours harbouring $G>A$ transitions or $G>T$ or $G>C$ transversions are positively associated with high linoleic acid intake. These associations are in line with the biological evidence (16,18) and are possibly the result of an increased formation of G-adducts resulting in $G>A$ transitions or $G>T$ or $G>C$ transversions. Increased intake of ω-6 PUFAs enhance tumorigenesis in experimental animals and *in vitro* systems by several mechanisms (47). The conjugated double bonds in PUFAs are highly sensitive to lipid peroxidation, and may generate fatty acid hydroperoxides. These hydroperoxides will be reduced by glutathione peroxidase to non-reactive fatty acid alcohols or may react with metal ions to yield alkoxyl radicals. The fatty acid alcohols do not lead to DNA damage and will not be discussed further. However, alkoxyl radicals are reduced to aldehydes, with 2,3-epoxy-4-hydroxynonanal (4-HNE) and MDA as the most prominent forms. The bifunctional alkylating agent 4-HNE can react with DNA to yield etheno and other base adducts which are thought to promote the carcinogenic process. Etheno-dG induces mainly transitions to A and transversions to T in bacteria (16,33,48,49) and etheno-dC induces transitions to T in the kidneys of monkeys (50). MDA is a major genotoxic carbonyl compound, which is also a by-product of the arachidonic acid metabolism in the synthesis of prostaglandins. MDA is mutagenic in bacterial and mammalian cells, and carcinogenic in rats (18). MDA reacts with DNA to form adducts, predominantly with deoxyguanosine to generate M_1G (15). The most common mutations in progeny molecules are $G>T$ transversions and $G>A$ transitions, with a minor contribution by $G>C$ transversions (16,18,19). In the current study, the associations with $G>A$ transitions, $G>T$ and $G>C$ transversions in the *K-ras* oncogene were only observed for colon cancer patients and not for rectal cancer patients. The reason for this apparent difference in the aetiology remains unclear. In addition, the observed significant association found for colon cancer could be a result of a type I error due to the

numerous associations analysed in this study. Although the studied associations were mainly hypotheses-driven, caution is warranted in interpreting these results and further investigations are needed.

Our results suggest that PUFA, and in particular linoleic acid, is an important dietary risk factor for colon tumours with *K-ras* mutations possibly by generating G > A transitions or G > T or G > C transversions in the exon 1 fragment of the *K-ras* oncogene. This implies that, for some dietary exposures like polyunsaturated fat intake, it is meaningful to account for somatic mutations in the *K-ras* oncogene in the aetiology of colon and rectal cancer.

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